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Characterization of metabolic profiles and lipopolysaccharide effects on porcine vascular wall mesenchymal stem cells

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## Abstract

The link between metabolic remodelling and stem cell fate is still unclear. To explore this topic, the metabolic profile of Porcine Vascular Wall Mesenchymal Stem Cells (pVW-MSCs) was investigated. At the 1<sup>st</sup> and 2<sup>nd</sup> cell passages, pVW-MSCs exploit both glycolysis and cellular respiration to synthesize ATP, but in the subsequent (3<sup>rd</sup> to 8<sup>th</sup>) passages they don't show any mitochondrial ATP turnover. Interestingly, when the 1<sup>st</sup> passage pVW-MSCs are exposed to 0.1 or 10 µg/mL lipopolysaccharides (LPS) for 4h, even if ATP synthesis is prevented, the spare respiratory capacity is retained and the glycolytic capacity is unaffected. In contrast, the exposure of pVW-MSCs at the 5<sup>th</sup> passage to 10 µg/mL LPS stimulates mitochondrial ATP synthesis. Flow cytometry rules out any ROS involvement in the LPS effects, thus suggesting that the pVW-MSC metabolic pattern is modulated by culture conditions via ROS-independent mechanisms.

Keywords: metabolic profiles; cellular acidification; lipopolysaccharide; porcine vascular wall mesenchymal stem cells.

## 1. INTRODUCTION

The Vascular Wall Mesenchymal Stem Cells are multipotent cells resident in vessels which show an intrinsic pro-angiogenic attitude to differentiate towards endothelial phenotype and to sustain the formation of a capillary network (Psaltis & Simari, 2015). Increasing evidence indicates that vascular stem cells play a fundamental role in angiogenesis and vascular regeneration after damage (Lu & Li, 2018).

Plasticity and adaptation, as well as a broad differentiation capacity, are relevant features of stem cells (L. Zhang et al., 2014). In order to preserve both stem cell potency and self-renewal capability, the maintenance of cellular homeostasis is a critical requirement. Metabolism gathers finely tuned biochemical pathways to match catabolic to anabolic events. Catabolic processes consist of breaking down/oxidation of nutrient-derived compounds (metabolites) to yield energy, while anabolism exploits energy to build macromolecules from simpler precursors. Noteworthy, the rapid stem cell proliferative potential is sustained by anabolic reactions, which synthesize macromolecules and biostructures. Conversely, differentiating cells enhance catabolism to fulfil the bioenergetic demand. Therefore, metabolic adaptations must obey to the specific requirements of diversified functions referable to cell status. A high metabolic flexibility is essential to balance anabolic and catabolic processes so as to adequately maintain the required cell features (Clifford D. L. Folmes, Dzeja, Nelson, & Terzic, 2012). The mitochondrial ATP production from oxidative metabolism is apparently ruled by the cell differentiation and mitochondrial status (C.D.L. Folmes, Dzeja, Nelson, & Terzic, 2012). Indeed, oxidative phosphorylation (OxPhos) re-oxidizes the NADH and FADH<sub>2</sub> coenzymes to preserve Krebs cycle activity and to provide the intermediates for fatty acids biosynthesis and *de novo* synthesis of aminoacids and nucleotides (Chandel, Jasper, Ho, & Passequé, 2016). Therefore, mitochondria play an essential role in homeostasis (Lees, Gardner, & Harvey, 2017).

An important question in stem cell metabolism is to identify the energy source, which sustains and addresses cell fate. The main nutrients utilized by stem cells are glucose and glutamine which are oxidized to produce ATP (Tohyama et al., 2016). Most likely, both glutaminolysis, which ensures the reduced coenzymes to fuel OxPhos and the intermediates for the synthesis of macromolecules and biostructures, and glycolysis, support anabolism for cell proliferation (Nesci, 2017). In general, stem cells prefer glycolysis to mitochondrial respiration, but the data on mesenchymal stem cell metabolism are still controversial (Shum, White, Mills, Bentley, & Eliseev, 2016). Probably, proliferation and self-renewal capability are fuelled by glycolysis-dependent anabolic pathways (J. Zhang, Nuebel, Daley, Koehler, & Teitell, 2012). Alternatively, the stemness features, irrespective of proliferative features, may be maintained by the low free radical production under conditions of poor mitochondrial oxidation. However, mitochondrial activity could also be independent of glycolytic regulation (Lisowski, Kannan, Mlody, & Prigione, 2018). Interestingly, during differentiation mesenchymal stem cells (MSCs) from bone marrow maintained glycolytic levels similar to undifferentiated cells (Shum et al., 2016). In addition, when MSCs use glycolysis to sustain metabolism (Chen, Shih, Kuo, Lee, & Wei, 2008), they exhibit a tubular mitochondrial network and a low ROS production (Forni, Peloggia, Trudeau, Shirihai, & Kowaltowski, 2016). The changes in cellular metabolism do not merely result from cell differentiation, but can also be required to allow survival under stress conditions. From this standpoint, the metabolic plasticity would also represent an adaptive strategy to cope with changing conditions. Porcine Vascular Wall Mesenchymal Stem Cells (pVW-MSCs) from the aorta of post-natal pig were isolated, due to the known relevance of swine as excellent model for translational studies (A. Zaniboni et al., 2014).

The present work explores the two major cell metabolic pathways, namely glycolysis and mitochondrial respiration, in pVW-MSCs at different cell passages and under stress conditions induced by lipopolysaccharide (LPS). LPS, naturally produced as component of cell wall by Gram-

negative bacteria, is known to exert profound effects on different stem cell types (Kukolj et al., 2018; Xing, Zhang, Jia, & Xu, 2019; Yin, Zhu, Wang, & Zhao, 2017). Being the role of metabolic changes and mitochondrial function and dynamics in stem cell differentiation still poorly understood (Shum et al., 2016), these studies represent an attempt to cast light on a promising topic, which opens the way to metabolic manipulations to address cell fate.

## 2. METHODS

### 2.1. Chemicals

Oligomycin mixture (A:B:C 64:15:17 %), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), antimycin A, rotenone, 2-deoxy-glucose (2-DG) were obtained from Vinci-Biochem (Vinci, Italy). 100X antibiotic-antimycotic solution (10,000 units/mL penicillin, 10,000 µg/mL streptomycin, 25 µg/mL Amphotericin B), Dulbecco's Modified Eagle's Medium (DMEM), M199, Fetal Bovine Serum (FBS) and CellROX® Deep Red Flow Cytometry Assay Kit were purchased from Life Technologies (Carlsbad, CA, USA). Trypsin-EDTA solution 1X, lipopolysaccharide (LPS) (*E. coli* 055:B5), and 0,4% Trypan Blue solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pericyte Growth Medium (PGM) was purchased from Promocell (Heidelberg, Germany).

### 2.2. Cell isolation and culture

pVW-MSCs were isolated and characterized from pig thoracic aorta as previously described (A. Zaniboni et al., 2014, 2015). The isolation method consists of two subsequent steps of chemical and physical selection. Briefly, cells were isolated from the media layers through enzymatic digestion and cultured overnight in DMEM 10% FBS plus 10X antibiotic-antimycotic solution in a 5% CO<sub>2</sub> incubator at 38.5°C. The day after, the culture medium was replaced by DMEM plus 10%FBS plus 1X antibiotic-antimycotic solution. Three days after, cells were serum starved overnight and then cultured in DMEM:M199 (1:1), 10% FBS, 1X antibiotic-antimycotic solution. Then cells were trypsinized, grown and expanded in PGM supplemented with 1X antibiotic-antimycotic solution and maintained at 38°C.

### 2.3. Lipopolysaccharide treatments

Before any treatment, cells were detached by trypsinization, counted in a Burker chamber and stained with 0.4% Trypan Blue to check viability. Aliquots of  $6 \times 10^5$  /mL alive cells ( $98 \pm 2\%$  viability) in PGM were seeded in micro-centrifuge tubes (1 mL) and maintained in incubator under Peltier thermostatisation at 38.5 °C and continuous stirring for 4 hours in the presence of the LPS doses to be tested ( 0, 0.1, 10 µg/mL LPS). The required LPS doses to attain the final concentrations were directly added to the medium.

### 2.4. Mitochondrial respiration

The oxygen consumption rate (OCR, nmol O<sub>2</sub>/min) ascribed to cell respiration was polarographically evaluated by Clark-type electrode using the Oxytherm System (Hansatech Instruments).

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3 OCR values were recorded after sequential addition of inhibitors to evaluate the different conditions  
4 under study. The ATP synthase inhibitor oligomycin was used to prevent mitochondrial ATP  
5 production and address cells to glycolysis. The ionophore FCCP was employed to uncouple  
6 mitochondria. Inhibition of the respiratory chain was obtained by the antimycin A plus rotenone  
7 mixture. Basal respiration was detected as baseline OCR before oligomycin addition. Minimal OCR  
8 was measured in the presence of 4  $\mu\text{g}/\text{mL}$  oligomycin, while maximal respiration was measured after  
9 addition of 0.5  $\mu\text{M}$  FCCP. Non-mitochondrial respiration was evaluated in the presence of 1  $\mu\text{g}/\text{mL}$   
10 antimycin A plus 4  $\mu\text{M}$  rotenone. The ATP turnover or oligomycin-sensitive respiration was obtained  
11 from the decrease in basal respiration after oligomycin addition. The difference between the maximal  
12 respiration and the basal respiration provided the spare capacity, which represents the ability to  
13 respond to an increased energy demand (Brand & Nicholls, 2011).  
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## 19 2.5. Cellular acidification

21 The glycolytic function was assayed by the colorimetric detection of L-lactate (Cayman's Glycolysis  
22 Cell-Based Assay Kit). This colorimetric assay, widely used for the determination of glycolytic  
23 efficiency, evaluates L-lactate, produced by the fermentation of pyruvate, the end product of  
24 glycolysis. The lactate dehydrogenase reaction produces NADH, which in turn yields a formazan dye,  
25 which absorbs at 490 nm. The formazan amount is proportional to that of L-lactate produced by pVW-  
26 MSCs. In each sample the L-lactate concentration was interpolated from the calibration straight line  
27 obtained by the detection of fresh L-lactate standards. By this method, the maximal glycolytic  
28 capacity was measured in the presence of 4  $\mu\text{g}/\text{mL}$  oligomycin, while the non-glycolytic acidification  
29 was detected in the presence of 15 mM 2-DG, a glucose analogue which cannot undergo glycolysis  
30 and blocks the glycolytic pathway. The glycolytic reserve was obtained as the difference between the  
31 L-lactate produced in the presence and in the absence of 4  $\mu\text{g}/\text{mL}$  oligomycin.  
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## 37 2.6. Oxidative Stress Evaluation by Flow Cytometry

38 In order to evaluate if LPS treatment is associated with oxidative stress in pVW-MSCs, CellROX®  
39 Deep Red Flow Cytometry Assay Kit was used following manufacturer's instructions. The cell-  
40 permeable CellROX® Deep Red reagent is essentially a non-fluorescent compound in the reduced  
41 state, but it exhibits a strong fluorogenic signal upon oxidation, thus providing a reliable measure of  
42 reactive oxygen species (ROS) in live cells. Blue-fluorescent, cell-impermeant SYTOX® Blue Dead  
43 Cell stain was used to discriminate alive from dead cells. Briefly, pVW-MSCs at 5<sup>th</sup> passage, were  
44 detached by trypsinization and aliquots of  $2 \times 10^5$  cells/mL were treated with 0  $\mu\text{g}/\text{mL}$  (Control, C)  
45 or 10  $\mu\text{g}/\text{mL}$  LPS (treated cells). As a positive control, cells were treated for 60 minutes with the  
46 common ROS inducer tert-butyl hydroperoxidase (TBHP). Stained samples were analyzed on  
47 MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) equipped with 638-nm  
48 lasers and 405-nm for the excitation of CellROX® Deep Red and SYTOX® Blue fluorescence,  
49 respectively. Fluorescence emission was collected using a 750LP filter and a 450/50BP for  
50 CellROX® and SYTOX® Blue, respectively. Data analysis from three independent experiments  
51 (n=3) was performed by Flowlogic Software (Miltenyi Biotec, Bergisch Gladbach, Germany).  
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## 58 2.7. Statistical analysis



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3 Statistical analyses were performed by SIGMASTAT software. One-way variance analysis  
4 (ANOVA), followed by Student-Newman-Keuls (SNK) test when  $F$  values indicated significance  
5 ( $P \leq 0.05$ ), was applied. The results are expressed as mean  $\pm$  SD.  
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### 8 9 10 3. RESULTS

11 The metabolic features of pVW-MSCs shifts from OxPhos to glycolysis and are remodelled through  
12 cell passages. Accordingly, in the early passages (1<sup>st</sup> and 2<sup>nd</sup>), cell respiration and ATP turnover are  
13 detectable and mainly attributed to OxPhos, which consumes 30% and 26% of oxygen for ATP  
14 synthesis at 1<sup>st</sup> and 2<sup>nd</sup> passages respectively. However, in the subsequent cell passages the metabolic  
15 pattern of prevailing cell respiration is not maintained (Fig. 1). At all cell passages pVW-MSCs  
16 exhibit the maximal respiration when stimulated by FCCP and the spare capacity attains positive  
17 values. This pattern indicates oxygen-sensitive mitochondria, maximally at the 5<sup>th</sup> passage (Fig. 1).  
18 LPS treatment differently affects pVW-MSCs, depending on the cell passage and on the LPS dose  
19 (Fig. 2). pVW-MSCs at the 1<sup>st</sup> passage treated for 4 hours with 0.1  $\mu\text{g}/\text{mL}$  and 10  $\mu\text{g}/\text{mL}$  LPS show  
20 an inhibited respiration. However, the inhibition of respiration is apparently removed by oligomycin.  
21 Indeed, OCR values show the typical response profile to FCCP and antimycin plus rotenone. The  
22 maximal respiration is decreased by both LPS treatments (Fig. 2A). Conversely, lactate production  
23 ascribed to glycolysis is not higher in LPS-treated pVW-MSCs than in control values, as it was  
24 expected to compensate the failed ATP production by OxPhos. Consistently, the glycolytic capacity,  
25 measured as difference in lactate production before and after oligomycin addition, is unaffected by  
26 LPS treatment being similar in all pVW-MSCs. Lactate production is inhibited by 2-DG both in LPS-  
27 treated and untreated pVW-MSCs (Fig. 2B). A different pattern is shown by the 5<sup>th</sup> passage pVW-  
28 MSCs, which do not show any ATP production apart from 10  $\mu\text{g}/\text{mL}$  LPS treated pVW-MSCs (Fig.  
29 2C). Oligomycin increases proton leak, namely the difference in OCR before and after oligomycin  
30 addition, in pVW-MSCs which do not perform any mitochondrial ATP synthesis. Irrespective of LPS  
31 treatment, FCCP stimulates cellular respiration, while the combination of the two inhibitors antimycin  
32 plus rotenone strongly inhibits the maximal oxygen consumption in all pVW-MSCs (Fig. 2C). Lactate  
33 production, which attains considerable levels both in untreated and 0.1  $\mu\text{g}/\text{mL}$  LPS-treated pVW-  
34 MSCs, drops in the 10  $\mu\text{g}/\text{mL}$  LPS-treated pVW-MSCs. Accordingly, in the 10  $\mu\text{g}/\text{mL}$  LPS-treated  
35 pVW-MSCs at the 5<sup>th</sup> passage a well detectable and oligomycin-sensitive ATP production (Fig. 2C)  
36 is associated with an high glycolytic capacity (lactate production in presence of oligomycin) (Fig.  
37 2D). As in all pVW-MSCs at the 1<sup>st</sup> passage (Fig. 2B), also at the 5<sup>th</sup> passage pVW-MSCs under all  
38 the conditions tested, L-lactate production is inhibited by 2-DG (Fig. 2D).

39 Flow Cytometry analysis shows that at the 5<sup>th</sup> passage the 10  $\mu\text{g}/\text{mL}$  LPS treatment for 4 hours is not  
40 associated to any significant increase in the production of ROS in pVW-MSCs. Accordingly,  $3.92\% \pm 1.62$   
41 LPS-treated cells show CellROX® Deep Red reagent strong fluorescent signal similar to the  
42 percentage of  $2.06\% \pm 0.33$  in control cells. After ROS induction promoted by TBHP treatment, high  
43 percentages of pVW-MSCs undergo oxidative stress, in contrast with the basal level of ROS shown  
44 both in control and in 10  $\mu\text{g}/\text{mL}$  LPS-treated pVW-MSCs (Fig. 3).  
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### 56 57 4. DISCUSSION

58 The elucidation of the mechanisms of metabolic switch at different cell passages can help to  
59 understand how the modulation of the mitochondrial function affects stem cell fate (Hopkinson et al.,  
60 2017). In spite of the wealth of studies, the mechanisms involved in the metabolic rearrangement



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3 under differentiation, widely observed in a variety of stem cells, are still poorly understood.  
4 Differentiation is usually featured by a metabolic shift from glycolysis to mitochondrial respiration  
5 and, conversely, reprogramming to “maximal stemness” is associated with a rise in glycolysis. There  
6 is a general consensus on the observation that the ratio glycolysis/mitochondrial oxidation affects the  
7 cell differentiation status. Undifferentiated cells also contain uncoupled and depolarized  
8 mitochondria (L. Zhang et al., 2014) and a basal level of reactive oxygen species (ROS), short-lived  
9 oxygen-containing molecules which increase during differentiation, thus consuming oxygen and  
10 increasing OCR values (L. Zhang et al., 2014).  
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14 The results obtained in the present study clearly show that the metabolic profile and the pVW-  
15 MSC responses to LPS treatment depend on the passage. As shown in Fig.1, pVW-MSCs at the 1<sup>st</sup> and 2<sup>nd</sup>  
16 passages depend on OxPhos to produce ATP, which has a mitochondrial origin. Accordingly, in 1<sup>st</sup>  
17 passage pVW-MSCs the increase in the 2DG-dependent lactate production in the presence of  
18 oligomycin (Fig. 2B) results from the activation of mitochondrial oxidative metabolism in the  
19 presence of a good glycolytic reserve. In the subsequent passages pVW-MSCs do not rely on  
20 mitochondrial respiration. The prominent spare respiratory capacity at the 5<sup>th</sup> passage (Fig. 1)  
21 indicates the pVW-MSC propensity to fulfil the energy requirements for differentiation and  
22 maturation and also suggests a low efficiency in metabolic reprogramming (Zhou et al., 2017).  
23 Apparently, LPS affects the mesenchymal properties of the undifferentiated pVW-MSCs, which have  
24 the ability to differentiate in vascular cells (Zaniboni et al., 2015). The mechanisms involved remain  
25 to be defined. LPS could inhibit the ATP synthase in pVW-MSCs at 1<sup>st</sup> passage in the absence of  
26 coupling. Accordingly, mitochondria in undifferentiated cells may be uncoupled even before the  
27 application of a chemical uncoupler (L. Zhang et al., 2014). However, since pVW-MSCs in presence  
28 of FCCP consume oxygen and are susceptible to the antimycin plus rotenone-mixture, irrespective of  
29 the treatment, they clearly exploit mitochondrial respiration (Fig. 2A). Noteworthy, at the 1<sup>st</sup> passage,  
30 glycolysis is apparently unaffected by LPS (Fig. 2B). Conversely, the metabolic profile shown by 10  
31 µg/mL LPS-treated pVW-MSCs at 5<sup>th</sup> passage is typical of mitochondrial oxidative activation (Fig.  
32 2C). Consistently, a low basal oligomycin-sensitive glycolysis is shown, while the glycolytic  
33 capacity, detected in the presence of oligomycin, which blocks the mitochondrial ATP synthesis, is  
34 apparently stimulated (Fig. 2C, D). Since any increase in apparent glycolytic capacity has been  
35 associated with cellular reprogramming and differentiation (Mookerjee, Nicholls, & Brand, 2016),  
36 this metabolic profile suggests that stress conditions induced by high LPS doses could stimulate  
37 differentiation. Under these conditions, the electron transport chain complexes, and especially  
38 complex I by reverse electron transfer (Robb et al., 2018), may enhance the formation of superoxide  
39 anion and other ROS. ROS, physiologically produced in mesenchymal stem cells by mitochondrial  
40 and extra-mitochondrial reactions, have a recognized role to address differentiation (Atashi,  
41 Modarressi, & Pepper, 2015). ROS generation could also explain the higher basal respiration in 10  
42 µg/mL LPS-treated pVW-MSCs with respect to both control and 0.1 µg/mL LPS-treated pVW-MSCs  
43 (Fig. 2C) (Piccoli et al., 2005). Differently from all the other conditions tested, the 5<sup>th</sup> passage  
44 10 µg/mL LPS-treated pVW-MSCs do not show any increase in OCR with respect to the basal  
45 respiration when the respiratory chain is blocked by the antimycin plus rotenone mixture (Fig. 2C).  
46 The unexpected slight increase in basal OCR may mirror a time-dependent increase in the rate of  
47 extra-mitochondrial oxygen-consuming reactions, including ROS formation (Piccoli et al., 2005).  
48 Accordingly, the OCR measured after antimycin plus rotenone mixture addition attains similar values  
49 under all the conditions tested and is unrelated to mitochondrial bioenergetics. However, quite  
50 unexpectedly, even if LPS is usually considered a powerful ROS inducer in mesenchymal stem cells  
51 (Yin et al., 2017), cytofluorimetric assays show that the bioenergetic changes in LPS-treated pVW-  
52 MSCs are clearly unrelated to ROS generation. LPS is known to interfere with stem cell signaling at  
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3 different levels (Yin et al., 2017) and to address lineage commitment (Kukolj et al., 2018; Xing et al.,  
4 2019). Probably, the highest LPS concentration tested, through ROS-independent mechanisms which  
5 remain to be defined, shifts pVW-MSC metabolism to cell respiration, even if the glycolytic capacity  
6 is maintained, to preserve the bioenergetic efficiency under unfavourable conditions. To sum up, if  
7 OxPhos is associated with differentiation and glycolysis with multipotency (L. Zhang et al., 2014),  
8 apparently high LPS doses, at least under the conditions adopted in the present study, promote in  
9 pVW-MSCs a metabolic rearrangement typical of differentiating cells which is apparently unrelated  
10 to ROS generation.  
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14 The metabolic modulation driven by LPS in pVW-MSCs may help to understand how local mediators  
15 and culture conditions can address cell fate. Moreover, since the pVW-MSC metabolic response to  
16 LPS depends on the passage, further studies may be addressed to cast light on the most suitable  
17 conditions for metabolic manipulations to be exploited in therapy.  
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## 20 21 CONFLICT OF INTEREST STATEMENT

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23 All authors declare they have no conflict of interest  
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## 58 FIGURE CAPTIONS

59 Figure 1. Bioenergetic profile of pVW-MSCs at different cell passages. The oxygen consumption rate  
60 (OCR), expressed as nmol O<sub>2</sub>/min/10<sup>6</sup> cells, was evaluated in the presence of oligomycin (to inhibit

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3 the ATP synthase), FCCP (OxPhos uncoupler) and antimycin plus rotenone (respiratory chain  
4 inhibitors), as described in Section 2, to determine the key respiratory parameters: *basal respiration*  
5 *ATP turnover*; *maximal respiration*; *spare capacity*. Data are the mean  $\pm$  SD (vertical bars) from three  
6 experiments carried out on different cell preparations.  
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11 Figure 2. LPS effects on pVW-MSC metabolism at 1<sup>st</sup> and 5<sup>th</sup> passages. Upper panel: mitochondrial  
12 respiration evaluated as oxygen consumption rate (OCR) and expressed as nmol O<sub>2</sub>/min/10<sup>6</sup> cells in  
13 1<sup>st</sup> A) and 5<sup>th</sup> passage cells C), respectively. Lower panel: glycolytic activity evaluated as L-lactate  
14 production in 1<sup>st</sup> B) and 5<sup>th</sup> passage cells D), respectively. Data expressed as points (A and C plots)  
15 and column chart (B and D plots) represent the mean  $\pm$  S.D (vertical bars) from three experiments  
16 carried out on different cell preparations. In B) and D) plots inhibitors were subsequently added: (■)  
17 no inhibitors; (■) +4  $\mu$ g/mL oligomycin; (■) +15 mM 2-DG; in each plot different upper-case letters  
18 indicate significantly different values among different LPS treatments under the same inhibiting  
19 conditions; different lower-case letters indicate significantly different values within each LPS  
20 treatment (0.0  $\mu$ g/mL, 0.1  $\mu$ g/mL, 10  $\mu$ g/mL LPS) ( $P \leq 0.05$ ).  
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27 Figure 3. Representative graphs of ROS evaluation by Flow Cytometry in pVW-MSCs at the 5<sup>th</sup>  
28 passage. 0.0  $\mu$ g/mL LPS (Control, C) for 4 hours (panel A), 10.0  $\mu$ g/mL LPS for 4 hours (panel B)  
29 and 50 mM TBHP for 60 minutes (Positive Control, panel C) before labeling by CellROX® Deep  
30 Red Flow Cytometry Assay Kit.  
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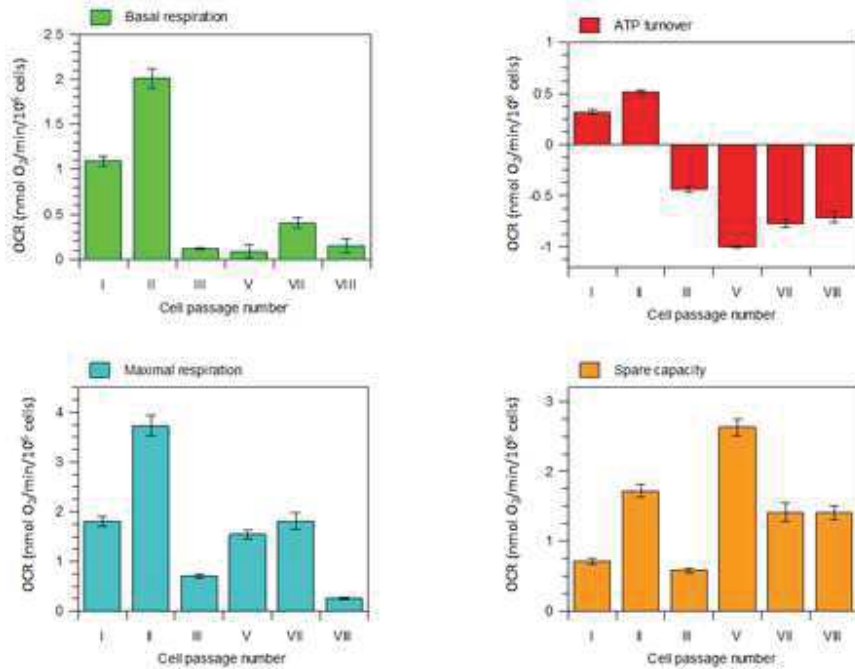


Figure 1. Bioenergetic profile of pVW-MSCs at different cell passages. The oxygen consumption rate (OCR), expressed as nmol O<sub>2</sub>/min/10<sup>6</sup> cells, was evaluated in the presence of oligomycin (to inhibit the ATP synthase), FCCP (OxPhos uncoupler) and antimycin plus rotenone (respiratory chain inhibitors), as described in Section 2, to determine the key respiratory parameters: basal respiration ATP turnover; maximal respiration; spare capacity. Data are the mean ± SD (vertical bars) from three experiments carried out on different cell preparations.

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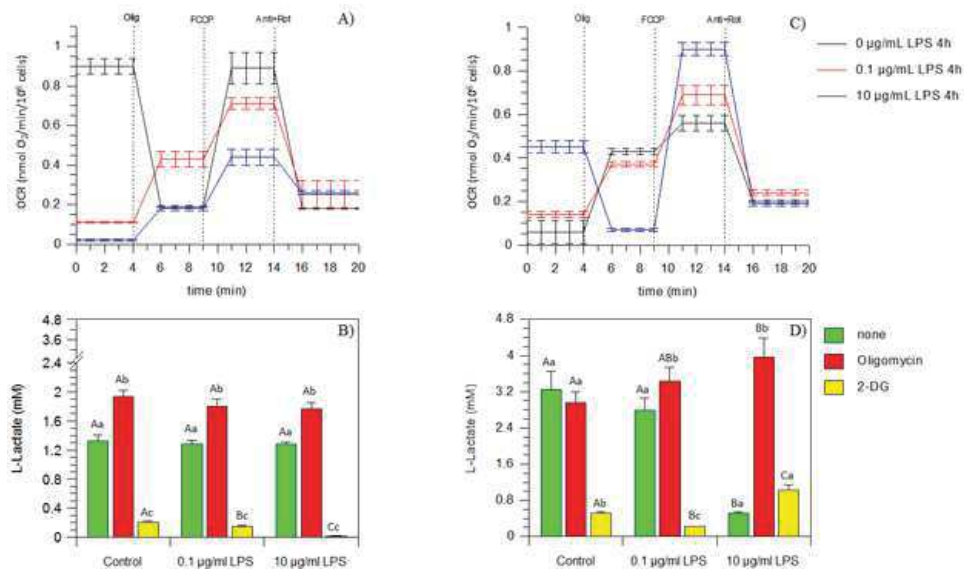


Figure 2. LPS effects on pVW-MSC metabolism at 1st and 5th passages. Upper panel: mitochondrial respiration evaluated as oxygen consumption rate (OCR) and expressed as nmol O<sub>2</sub>/min/10<sup>6</sup> cells in 1st A) and 5th passage cells C), respectively. Lower panel: glycolytic activity evaluated as L-lactate production in 1st B) and 5th passage cells D), respectively. Data expressed as points (A and C plots) and column chart (B and D plots) represent the mean  $\pm$  S.D (vertical bars) from three experiments carried out on different cell preparations. In B) and D) plots inhibitors were subsequently added: (green) no inhibitors; (red) +4  $\mu$ g/mL oligomycin; (yellow) +15 mM 2-DG; in each plot different upper-case letters indicate significantly different values among different LPS treatments under the same inhibiting conditions; different lower-case letters indicate significantly different values within each LPS treatment (0.0  $\mu$ g/mL, 0.1  $\mu$ g/mL, 10  $\mu$ g/mL LPS) ( $P \leq 0.05$ ).

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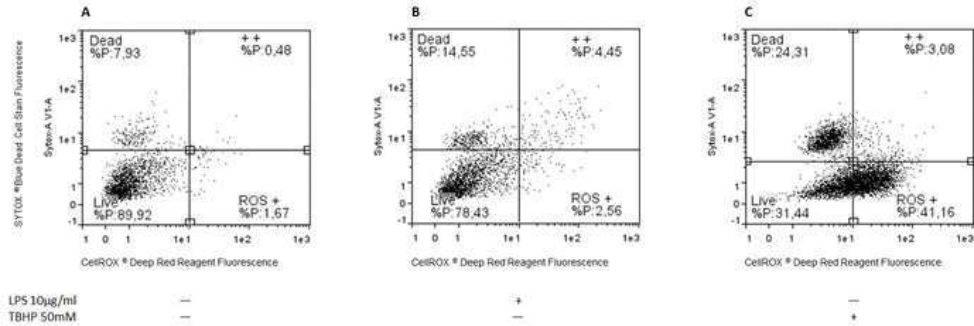
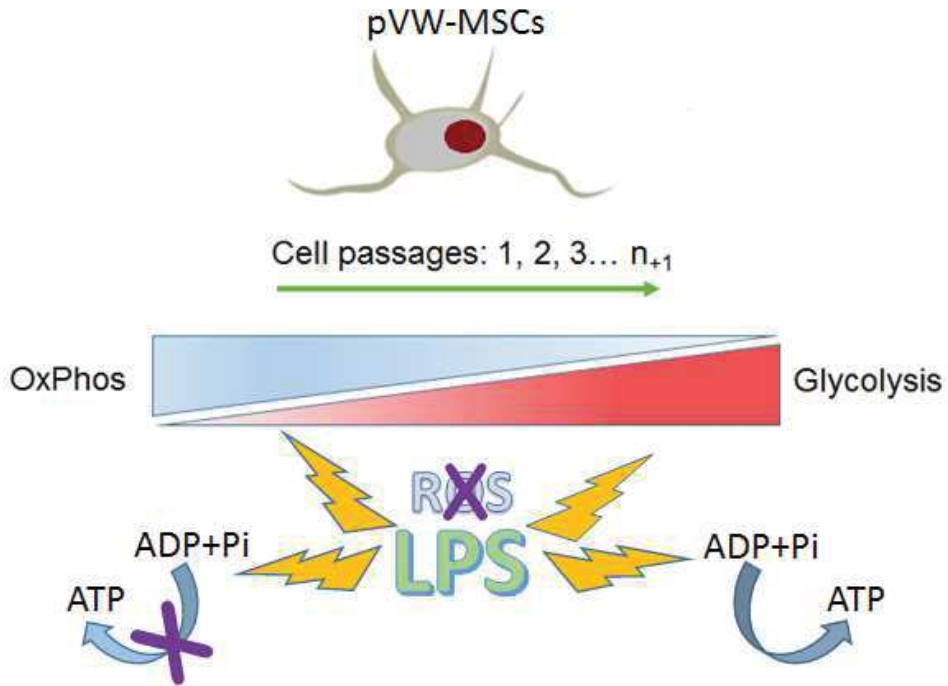


Figure 3. Representative graphs of ROS evaluation by Flow Cytometry in pW-MSCs at the 5th passage. 0.0 µg/mL LPS (Control, C) for 4 hours (panel A), 10.0 µg/mL LPS for 4 hours (panel B) and 50 mM TBHP for 60 minutes (Positive Control, panel C) before labeling by CellROX® Deep Red Flow Cytometry Assay Kit.

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Graphical Abstract

50x40mm (300 x 300 DPI)